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Pulsed-Field Gel Electrophoresis as a Replacement for Bacteriophage Typing of *Staphylococcus aureus*

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Bacteriophage typing (BT) (World Health Organization method) has been used at the Centers for Disease Control and Prevention for over 30 years to type isolates of *Staphylococcus aureus*. Since studies have shown that BT patterns have poor reproducibility and because BT fails to type a high percentage (15 to 20%) of isolates, the Centers for Disease Control and Prevention has converted from using BT to using pulsed-field gel electrophoresis (PFGE) for strain typing *S. aureus*. We compared the results of BT with results of PFGE for typing 300 isolates of *S. aureus*, including strains from several well-characterized outbreaks. Ninety-six isolates were BT group I, 19 were group II, 82 were group III, 7 were group V, and 96 were nontypeable. PFGE identified subgroups within each phage group and thus was more discriminating than BT, which identified no subgroups. PFGE was able to type all isolates and distinguish related from unrelated strains of *S. aureus*. Our modified, standardized PFGE methodology should enable typing laboratories to obtain rapid, reliable results in 3 to 4 days when starting with an isolated colony on agar media.

Outbreaks of infections caused by *Staphylococcus aureus* continue to be a problem for health care facilities (16). Identifying strains among the outbreak isolates is a major step in determining the source of the outbreak and in designing subsequent control measures. Two typing techniques frequently used for strain delineation of *S. aureus* are bacteriophage typing (BT) (4, 14) and pulsed-field gel electrophoresis (PFGE) (7, 8, 10, 15). The Centers for Disease Control and Prevention (CDC) has used BT for over 30 years to discriminate among strains of outbreak-related *S. aureus*. Unfortunately, this technique has several weaknesses: (i) it characterizes isolates on the basis of a phenotypic marker that has poor reproducibility; (ii) it does not type all isolates; and (iii) it requires maintenance of a large number of phage stocks and propagating strains, which confines its use to relatively few reference laboratories. PFGE is a molecular typing technique that does not have the same limitations as BT, since the bacterial genome is more stable than most protein markers. PFGE offers advantages over other DNA-based *S. aureus* strain-typing techniques (9, 12). For example, plasmid typing offers only moderate reproducibility, restriction fragment length polymorphism typing requires the analysis of complex banding patterns, and ribotyping is highly labor-intensive and time-consuming. To date, all *S. aureus* isolates are typeable by PFGE; however, criteria for PFGE banding pattern interpretation have not been standardized despite several attempts (9, 11). In addition, PFGE techniques currently being used are technically demanding.

This paper presents the data that served as the basis for moving from BT to PFGE as the method of choice for strain typing *S. aureus* isolates at the CDC. During this investigation, we developed (i) a shorter and easier standardized method for typing *S. aureus* by PFGE, (ii) the interpretation of the resulting banding patterns used at the CDC, and (iii) the criteria for submitting outbreak isolates of *S. aureus* to CDC for PFGE analysis.

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MATERIALS AND METHODS

Bacterial isolates. A total of 300 isolates of *S. aureus* were selected for inclusion in this study. Ninety-six isolates were BT group I, 19 were group II, 82 were group III, 7 were group V, and 96 were nontypeable. Phage group I contained cultures representing 16 outbreak clusters; isolates having a phage pattern of 80/81 or 52/52A/80/81 were placed into two clusters because of evidence supporting their close relationship (1, 2, 5). Phage groups II, III, and V contained cultures representing four, seven, and one outbreak cluster(s), respectively. These selected isolates were taken from a collection of strains that were sent to CDC for phage typing from a variety of hospitals located in the United States over a period of 35 years.

BT. Phage typing was performed as described previously by using the international bacteriophage typing set at the routine test dilution and 100 times the routine test dilution (3). Outbreak strains that differed by the presence or absence of one phage were considered related; strains, which differed by the presence or absence of two or more phages were considered unrelated.

PFGE. The protocol for the preparation of chromosomal DNA was modified from that described by Goering and Winters (6). The cultures were grown overnight in 5 ml of brain heart infusion broth, and 0.7 ml of this overnight culture was harvested by centrifugation (7,000 rpm for 2 min [Eppendorf model 5415C]) in a microcentrifuge tube. Cells were washed once in 1 ml of autoclaved TEN buffer (0.1 M Tris Cl, 0.15 M NaCl, 0.1 M EDTA) and centrifuged again. The washed cells were resuspended in 0.3 ml of autoclaved EC buffer (6 mM Tris Cl, 1 M NaCl, 0.1 M EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% Sarkosyl). Two microliters of a 1-mg/ml solution of lysostaphin (Ambicin L; Applied Microbiology, New York, N.Y.) dissolved in 20 mM sodium acetate was added to the cell suspension, and the mixture was subsequently vortexed. Three hundred microliters of 2% SeaPlaque agarose (FMC Corp., Rockland, Maine) dissolved in EC buffer was added to the lysostaphin-cell suspension. The suspension was briefly vortexed and quickly pipeted into a plug mold. The plug was allowed to solidify at room temperature for about 10 min. After solidification, the plug was placed in a tube (16 by 100 mm) containing 3 ml of EC buffer and the cells in the plug were lysed for 1.0 h at 37°C without shaking. After the lysing step, the EC buffer was removed and replaced with 3 ml of autoclaved TE buffer (10 mM Tris Cl, 5 mM EDTA) and the tube was incubated for 1 h at 55°C without shaking. The plug was then transferred to 3 ml of fresh TE buffer for storage at 4°C until further analysis.

For electrophoresis, the plug was cut into small slices (2 by 5 mm) and placed in a 125-μl total restriction enzyme mixture (restriction buffer plus sterile distilled water) containing 20 U of *Sma*I (New England BioLabs, Beverly, Mass.). After a 2-h incubation at 25°C with shaking at 140 rpm, chromosomal restriction fragment patterns were analyzed by loading the trimmed slices of the plug into a well of a 1% SeaKem agarose running gel (FMC Corp.). The running gel was prepared in 0.5× TBE buffer (Bio-Rad, Richmond, Calif.). The wells containing the plug slices were sealed with 0.8% SeaPlaque agarose. Electrophoresis was performed with the CHEF-DR II or CHEF-DR III electrophoresis cell (Bio-Rad, Melville, N.Y.). Bacteriophage lambda DNA concatemers (Bio-Rad) were used as size standards and served as a control for the running parameters of the CHEF-DR units. The running parameters were as follows: initial pulse, 5 s; final

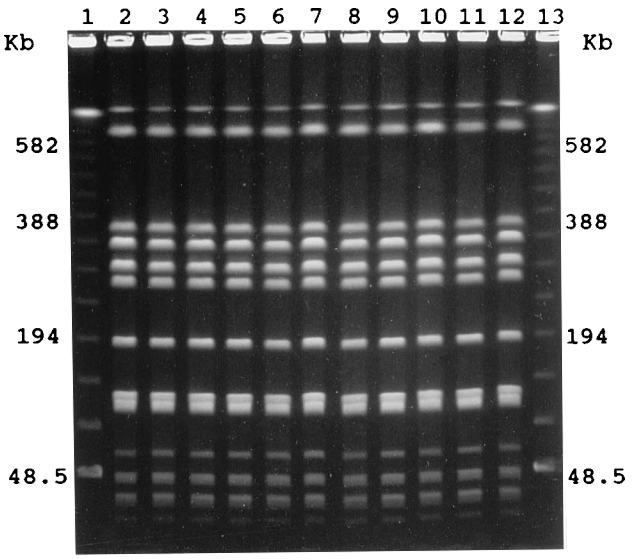

FIG. 1. PFGE patterns from an outbreak of BT-nonreactive isolates. Molecular size standards (lambda oligomers) are in lanes 1 and 13. Lanes 2 to 12 each contain a different isolate.

pulse, 40 s; voltage, 200 V or 6 V/cm; time, 20 h; and temperature, 12 to 14°C. The gels were stained with ethidium bromide and photographed.

Interpretation of PFGE banding patterns. The interpretation of the banding patterns was done visually according to the following guidelines: (i) banding patterns from the majority of epidemiologically related isolates that appeared identical in size and number of bands were considered to represent the same strain (modal pattern), which was designated strain A; (ii) isolate banding patterns that differed from the main pattern because of one or two genetic events (three or fewer band differences due to a mutation, insertion, deletion, and/or inversion) were considered those of a subtype within the main group (e.g., A_1, A_2, and A_3, etc.); and (iii) isolate banding patterns that differed from the main pattern by four or more bands which could not be explained by at most two genetic events were considered those of a different strain (e.g., designated strains B, C, and D, etc.).

RESULTS

Typeability. Ninety-six (32%) of the 300 isolates of *S. aureus* were nonreactive by BT. Eighty-three of the 96 isolates were isolated from three different outbreak clusters. Because of the inability of BT to type these isolates, no usable information was available to enable infection control personnel to confirm the occurrence, or support the resolution, of an outbreak. PFGE was able to show strain relationships within two of the outbreaks. The third outbreak cluster was found by PFGE to contain two different strains. Figure 1 displays the PFGE banding patterns of outbreak isolates that were nonreactive by BT. The uppermost band in this figure is not undigested DNA, but a large piece of DNA that has no *Sma*I restriction site.

Discriminatory power. Discriminatory power refers to the ability of a technique to differentiate among unrelated strains. A comparison of the abilities of BT and PFGE to differentiate among isolates representing phage groups I, II, III, and V is given in Table 1. The number of patterns for BT and PFGE was determined by analyzing each outbreak independently.

(i) Phage group I. Representatives of phage group I included 15 outbreak cluster isolates from a large variety of geographic locations and sources. For the 96 isolates studied, BT produced 18 patterns and PFGE produced 15 type patterns and 31 subtype patterns. Strain-typing agreement occurred between the two typing techniques for all outbreaks except two. For one of these two outbreaks (outbreak 1 in Table 2), BT gave three patterns, 29/52/80/81, 29/52/95, and 29, indicating three strains were present. For the same outbreak, PFGE showed one type pattern and one subtype pattern, indicating the presence of one strain. For the second outbreak, BT showed phage types of 29/52/79/80 and 29/80, indicating two strains were present. PFGE results showed one type pattern and one subtype pattern, indicating one strain was present. Comparison of the different outbreak patterns revealed two predominant or main PFGE patterns for this phage group. This finding suggests that a small number of clones constitute phage group I. Further studies are under way to help elucidate these results. All of the subtypes were found to have one of the two main patterns. Lanes 2 and 3 of Fig. 2 show the main representatives of phage group I; lane 2 shows the predominant pattern.

TABLE 1. Comparison of the discriminatory powers of BT and PFGE

Phage group	No. of isolates	No. of outbreaks[a]	Total no. of phage patterns[b]	Total no. of PFGE patterns[b]	
				Types	Subtypes
Nontypeable	96	7	0	8	8
I	96	15	18	15	31
II	19	4	4	4	2
III	82	7	15	11	13
V	7	1	1	1	5

[a] Number of presumed outbreak-related clusters as determined by epidemiologic data.
[b] Total number of patterns for BT and PFGE was determined by analyzing each outbreak independently.

(ii) Phage group II. Representatives of phage group II included isolates from four outbreak clusters and four different geographic locations. For the 19 isolates studied, BT produced four patterns and PFGE produced four type patterns and two subtype patterns. For outbreak 2 (Table 2), BT included a nonoutbreak isolate as part of the outbreak. Each cluster of isolates from the four sets gave a different main PFGE pattern, with two of the main patterns showing a subtype or slight variation. Three of the four main patterns are shown in Fig. 2 (lanes 4 to 6).

(iii) Phage group III. Representatives of phage group III included isolates from five different geographic locations and seven different outbreak clusters. For the 82 isolates studied, BT showed 15 patterns and PFGE produced 11 type patterns and 13 subtype patterns. Strain-typing agreement between the two techniques occurred for all outbreaks except three. For one of these three outbreaks, BT gave two phage types of 53/77 and 53/54/75/77/95, indicating two strains were present. PFGE results showed the presence of one strain by giving a type pattern for isolates having the phage pattern of 53/54/75/77/95 and a subtype pattern for isolates having the phage type of 53/77. For the second outbreak, PFGE results showed 3 type patterns and 11 subtype patterns, indicating the presence of three strains. BT gave results indicating that there were six strains. The first of the three strains for PFGE consisted of phage types 75 (BT strain 1), 75/77 (BT strain 1), and 47/81 (BT strain 2). The second PFGE strain consisted of phage types 47/54/75 (BT strain 3), 47/54/75/77 (BT strain 3), 47/54/75/81 (BT strain 3), 47/54/75/77/81 (BT strain 3), 6/47/54/81 (BT strain 4), and 53 (BT strain 5). The third PFGE strain consisted of the phage type 47/53/54/75/77/83A (BT strain 6). PFGE showed 7 main patterns (Fig. 2, lanes 7 to 13), with the 13 subtype patterns falling under 2 of the 7 main patterns (lanes 8 and 9). BT gave a wide variety of different phage combinations. For the third outbreak (outbreak 3 in Table 2),

TABLE 2. Epidemiologic application of PFGE to representative outbreaks of *S. aureus* infection

Outbreak	Phage group	No. of isolates[a]	No. of outbreak-related isolates	No. of different patterns			No. correctly classified by[c]:		No. misclassified by BT[d]
				Expected	By BT	By PFGE[b]	BT	PFGE	
1	I	6	6	1	3	1	4	6	2
2	II	8	7	2	1	2	7	7	1
3	III	17	14	4	5	4	13	14	1
4	III	13	12	2	3	2	11	12	1
5	V	7	7	1	1	1	7	7	0

[a] Total number of outbreak cluster isolates submitted for strain analysis.
[b] Number of major PFGE patterns, excluding subtypes.
[c] Number of isolates correctly classified as outbreak related.
[d] Outbreak isolates were classified as unrelated for outbreaks 1, 3, and 4, and one nonoutbreak isolate was classified as related for outbreak 2.

BT gave five different patterns and PFGE gave four main patterns with one subtype. BT excluded an outbreak isolate from the outbreak-related cluster.

Figure 3 demonstrates the usefulness of PFGE in distinguishing related from unrelated isolates in a food outbreak (outbreak 4 in Table 2). Nine of the 13 isolates were found to be the same strain, designated strain A, by PFGE (Fig. 3, lanes 2 to 8, 11, and 12). Isolates in lanes 9, 10, and 13 differ by one band (around 48 kb) from strain A; therefore, they are subtypes. This band difference may be attributable to the loss of a plasmid in the subtype isolates. The final isolate was designated strain B by PFGE because of the completely different banding pattern (Fig. 3, lane 14). Among the nine strain A isolates and three subtypes, BT gave a pattern of 47/54/75 for all except one strain A isolate that was nonreactive. Strain B was phage type 95 by BT.

(iv) Phage group V. The isolates tested for phage group V came from one outbreak. For the seven isolates, BT gave one pattern, 94/96. For PFGE, there was only one main pattern, with the remaining isolates being classified as subtypes (Fig. 4). The isolates in lanes 2 and 5 of Fig. 4 have the same PFGE pattern. On the basis of the CDC criteria for PFGE banding pattern interpretation, these two isolates were classified as belonging to the same strain, designated strain A. The other isolates are subtypes of strain A and were classified as subtypes A_1 to A_5. As shown in Table 2 (outbreak 5), both techniques were correct in identifying all isolates as outbreak related.

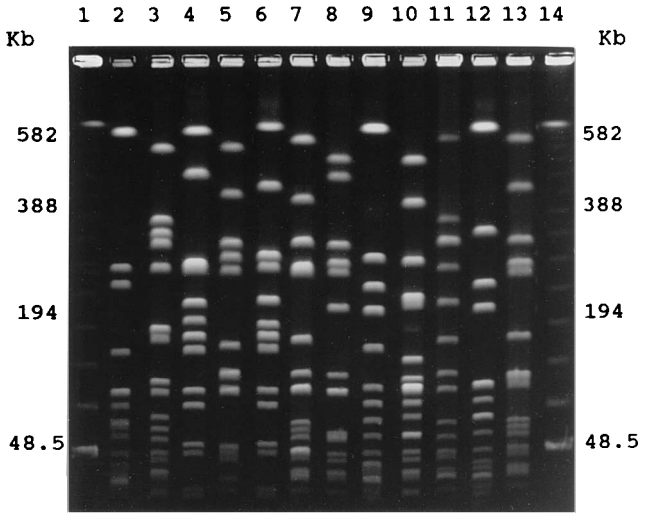

FIG. 2. Main PFGE patterns for the isolates found to belong to phage groups I, II, and III. Representatives of phage group I are in lanes 2 and 3. Representatives of phage group II are in lanes 4 to 6. Representatives of phage group III are in lanes 7 to 13. Molecular size standards (lambda oligomers) are in lanes 1 and 14.

DISCUSSION

Strain identification is an integral part of outbreak investigations. For over 30 years, the CDC, like other large reference laboratories around the world, has been using BT as its strain-typing method. However, this technique has several reported problems, including problems with typeability, reproducibility, and the labor required. The goal of this study was to determine if PFGE is a more epidemiologically accurate strain-typing method than BT for *S. aureus*. Our studies found two main advantages of using PFGE: (i) it had universal *S. aureus* strain-typing ability, and (ii) it was better than BT at distinguishing epidemiologically related strains from unrelated strains.

After PFGE was established as the method of choice, it was necessary to develop a simple procedure that could provide rapid, reliable results. The procedure described here can be completed within 3 to 4 days, starting with an isolated colony on agar media. The main difference between this procedure and other published PFGE procedures is the lack of a proteinase step. Unlike that of some genera, staphylococcal DNA can be released from intact cells in agarose plugs, restricted with endonucleases, and electrophoresed before significant DNA

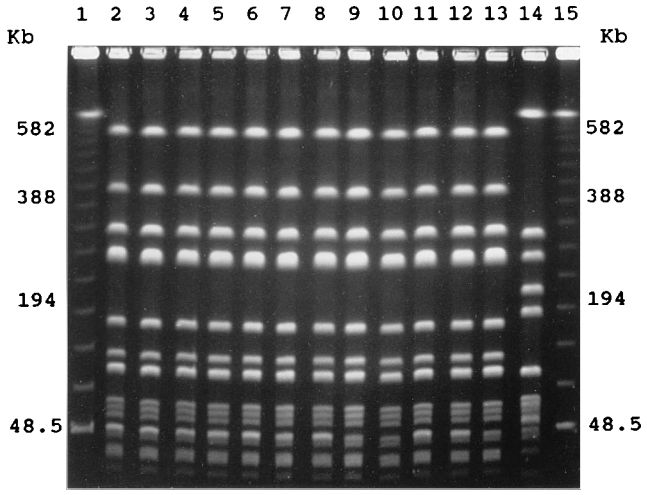

FIG. 3. PFGE patterns from food outbreak isolates. Molecular size standards (lambda oligomers) are in lanes 1 and 15. Lanes 2 to 14 each contain a different isolate.

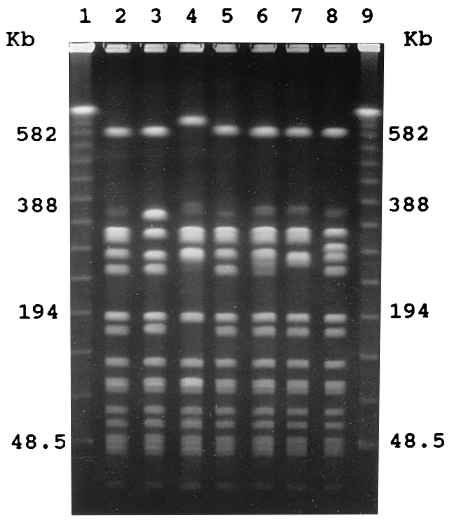

FIG. 4. PFGE patterns from outbreak isolates belonging to phage group V. Molecular size standards (lambda oligomers) are in lanes 1 and 9. Lanes 2 to 8 each contain a different isolate.

degradation takes place. However, if one is using a restriction endonuclease that requires overnight digestion, a proteinase or similar step may be necessary because restriction incubation temperatures of 25 to 37°C allow the resident nucleases to cleave DNA. The stability of the DNA in the plugs will differ from strain to strain. Some plugs (prior to restriction endonuclease digestion) have been kept in TE buffer for 3 to 6 months without any noticeable DNA degradation, while the DNA in other plugs has shown degradation after 1 month. Since a critical process in PFGE is the lysis of the isolate, the use of the recombinant lysostaphin Ambicin L is an essential component of our protocol. This protocol can also be applied to the coagulase-negative staphylococci with two modifications: (i) an increase in the lysostaphin amount from 2 to 10 μl and (ii) an increase in the lysing time from 1 to 2.0 or 2.5 h, depending upon the species. Further optimization of PFGE for strain typing staphylococci is currently under way. This technique, unlike BT, does not require the time-consuming maintenance of a large number of stocks. The cost per PFGE assay of 13 isolates is around $36, excluding technician time and the initial cost of equipment. The total price consists of the costs for reagents, $15; media, $10; glassware, $9; and miscellaneous items (film), $2. In comparison, the total cost for BT is about $10 per isolate. The disadvantage of this technique in comparison with BT is the high cost of the capital equipment for PFGE.

A final goal of this study was to provide to health institution personnel interested in having strains of staphylococci analyzed by PFGE at the CDC criteria for submission of isolates. The criteria are as follows: (i) testing will be performed only on outbreak-related isolates submitted in pure culture on Trypticase soy agar slants (or equivalent); (ii) all isolates must be sent in one shipment, since accurate interpretation requires that all isolates from an outbreak be tested on the same gel, if possible; (iii) cultures should be sent to the CDC through the state health department with a letter from the requestor explaining the epidemiologic need for PFGE analysis, the demographics of the outbreak, and locally obtained testing results (e.g., antimicrobial susceptibility); and (iv) if hospitals have had a previous problem with *S. aureus*, they are encouraged to submit one or two historical control strains to determine whether the same strain is responsible for the outbreak.

The results from PFGE analysis must always be analyzed in light of the information from the epidemiologic investigation. There are four main categories into which isolates may fall when the interpretation guidelines presented herein are used (13). (i) Isolates with identical banding patterns (same number and size of bands) will be considered "indistinguishable" and if epidemiologically related will be considered the same strain (i.e., designated strain A); (ii) isolates with banding patterns differing from that of strain A as a result of one genetic event will be considered "probably related" to that main strain and will be considered subtypes if the epidemiologic data suggest that they are linked to the outbreak; (iii) isolates with banding patterns differing from that of strain A as a result of two genetic events will be considered "possibly related" to strain A and will be considered subtypes if the epidemiologic information suggests that they are involved in the outbreak; and (iv) isolates with banding patterns differing from that of strain A as a result of three or more genetic events will be considered "different" strains (i.e., designated strain B, C, or D, etc.).

Because of the advantages PFGE has over BT, including greater discriminatory power, universal typeability, and ease of use and interpretation, the CDC has converted to using PFGE in epidemiologic investigations of *S. aureus*. The PFGE method described herein should enable typing laboratories to obtain rapid, reliable results when investigating staphylococcal outbreaks.

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